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COE Annual Report 07-08

Center of Excellence for Therapeutic Individualization for Breast Cancer

Annual Report: General Overview

George W. Sledge, Jr. M.D. Principal Investigator

The report submitted herein includes reports from subcontractors involved in the COE, which explain in detail the efforts of the COE during the past year. This report will highlight the overall progress made by the Center of Excellence.

As discussed in a recent teleconference between the COE team and DOD representatives, the past year has seen a significant change in our overall approach to the Center of Excellence for Therapeutic Individualization for Breast Cancer. Our initial approach, as originally set out, involved obtaining frozen tissue samples for all women entering the trial. Obtaining these tissues in a timely fashion proved more difficult than we initially predicted, due to regulatory issues that delayed trial participation and due to accrual problems at clinical sites. Simultaneously, changes in technology have made it possible to perform high-quality analyses on formalin-fixed, paraffin-embedded tissues. We therefore shifted the focus of clinical trial material from fresh frozen tissues to formalin-fixed, paraffin-embedded tissues (FFPET). This shift in focus has had important consequences for trial performance, accrual, and technology.

From a trial performance standpoint, we inaugurated during the past year our COE05 trial. This trial, our "Retrospective-Prospective" trial, has obtained FFPET samples from women who have died of metastatic breast cancer and on whom clinical response and time to treatment failure data were available. To date samples from over 60 patients have been obtained. Because patients may have been exposed to more than one chemotherapy regimen during the course of their disease, patients can provide informative data for multiple agents. We are also currently in negotiation with a prominent medical oncology consortium in Poland (headed by Dr. Jacek Jassem) to obtain additional specimens for COE05; at present this consortium has identified ~200 patients who are case-eligible for COE05. Together with our current samples, these additional samples should provide us sufficient material to create multiple drug-specific signatures.

Similarly, we have seen an increase in accrual to our parent COE01 trial during the past year, with more than 40 patients having been accrued to date. Accrual for both COE01 and COE 05 continues. It is expected that, taken together, COE01 and COE05 will provide complimentary data sets for developing and validating prognostic signatures.

In addition to patients accrued through COE01 to date, the Center has also had the good fortune to identify other available tissue sets obtained through previously performed clinical trials. Predominantly these represent tissues obtained by Dr. Jenny Chang and her colleagues at Baylor University, and include patients treated in the neoadjuvant setting with doxorubicin plus cyclophosphamide (AC) or docetaxel. These sample sets, in aggregate, amount to approximately 300 patients. Because docetaxel was not in out original proposal, we will amend COE01 to include analysis of this agent. We will also, because the AC samples obtained in this fashion satisfy our requirements for this combination, close this arm of the COE01 study.

From a technology standpoint, the use of FFPET has important implications. FFPET, as a result of fixation, alters tissues used for analysis of proteins and mRNA. In particular, for mRNA, degradation of message represents a significant challenge. At the time of initiation of this project, available technology was inadequate to rise to this challenge. Advances in techniques directed at this problem have been rapid, and COE investigators (as outlined in the scientific core sections of this update) have taken important steps to utilize the new technology. In particular, this work has culminated in a recent publicationin Biotechniques entitled "Optimization of RNA extraction from FFPE tissues for expression profiling in the DASL assay" that has developed the techniques to be used for FFPET in this trial.

Given the large amount of tissue rapidly becoming available to the Center, we expect the next year to be one of substantial progress towards our goal of obtaining drug-specific signatures for therapeutic individualization in patients receiving therapy for metastatic breast cancer. We wish to thank the DOD for its continuing support and useful recommendations regarding the Center.

Proteomics Core:
The proteomics core received 22 Pretreatment serum samples. Nine were from the AC arm of COE01 and 13 were from the Gemcitabine arm of the Project. We fractionated these 22 samples on a cation exchange column and created 6 protein fractions. Serum specimens for each patient were loaded onto the ion exchange column at pH 9.0, and what passed through the column was designated as fraction 1. The column was then washed with the loading buffer, and sequentially eluted with buffers set to pH 7.0, 5.0, 4.0, and 3.0. Proteins eluting from the columns at each of these pH's were designated as fraction 2 - pH 7.0; fraction 3 – pH 5.0; fraction 4 – pH 4.0; fraction 5 – pH 3.0. The column was subsequently eluted with a somewhat hydrophobic solution consisting of 33.3% isopropanol/16.7% acetonitrile/0.1%Trifluoroacetic acid.

Fractions 1, 4, 5, and 6 were analyzed for their proteomic signature by SELDI-TOF mass spectrometry because these fractions contained the most diverse populations of proteins. Samples were run in duplicate on the IMAC30 (metal binding) chips loaded with copper ion, CM10 chips (carboxymethyl ion exchange matrix), and H50 chips (hydrophobic). The individual chips were loaded with sinapinic acid (SPA) as the energy absorbing molecule, and the instrument was configured to detect proteins in the mass range of 2,000-170,000 daltons. Individual patient spectra generated during the analysis was normalized prior to employing the peak detection algorithm and performing a univariate analysis of the spectral data in order to identify specific protein components within the serum of these patients that could distinguish the patients on the AC arm from those on the Gemcitabine arm of COE01.

Conclusions:
The results of this effort suggested to us that we would not be able to distinguish the patients within these two groups from one another; using the major peaks identified by the peak detection algorithm. However, this may in fact be precisely what we should see in this cohort of patients at this point in time. To convince myself of this, I would like to continue the analysis part of this investigation by altering some of the search parameters used in this initial comparison of molecular signatures; in the hopes of identifying either consistencies within individual patient spectra that could be used to distinguish between these two groups of patients, or differences that under more relaxed criteria, would reveal a distinguishing feature. It should be important to note that it is formally possible that the major serum component signatures being detected in each of the analyzed fractions are in fact generated as a general cellular response to exposure to cytotoxic chemotherapy; regardless of the drug mechanism employed. Since both approaches target, at the very least, the DNA synthetic DNA repair process, it is not unreasonable to expect that the major components released into serum are associated with the cellular response to these cytotoxic reagents, and thus do not show any clear proteomic signatures distinguishing the two arms of this trial. It may be more productive for us to consider searching for signatures associated with those patients who are responding to the treatments vs. those who are not responding to the corresponding treatment or perhaps to even any of these treatment protocol arms.

Breast cancer and intrinsic chemoresistance

Baylor University

ABSTRACT

Chemotherapy treatments are initially effective in controlling breast cancer. Yet, many women relapse and sadly, die from their disease, even if they had originally responded to treatment. In the U.S, more than 40,000 women this year alone will relapse, and standard therapies today only can palliate and prolong lives but cannot eradicate the disease. There is increasing evidence that standard treatments like chemotherapy just kills the dividing "daughter" (progenitor) cells, without killing the cancer stem cell that does not divide or die. This is especially relevant for young women, as they have "aggressive" cancers that divide, and although current therapies can stop the dividing daughter progenitor cancer cells, women still relapse because the chemotherapy has not affected or killed the therapy-resistant cancer stem cells, with the ability, when activated, to give rise to many daughter cells of high proliferative potential. Analogous with the propensity of dandelion roots to regenerate weeds, re-growth of tumors from this *intrinsically resistant* sub-population has been termed “the dandelion hypothesis”. This hypothesis provides a unified explanation for the success and failures of cytotoxic chemotherapy - namely, although the majority of cells in the original tumor may be killed, the most important target, a small population of therapy-resistant cancer cells possessing the capacity to form new cancers is spared.

Recently, a small sub-population of breast cancer stem cells, labeled as $CD44^{+}/CD24^{-/low}$, was isolated. We have shown that chemotherapy reduces the overall size of the tumor, but spares this rare sub-population of cells, labeled as $CD44^{+}/CD24^{-/low}$, which increases proportionately as the bulk of the tumor decreases. We have also shown that these $CD44^{+}/CD24^{-/low}$ cells are capable of forming cancers in culture medium (called mammospheres), and new cancers in immunocompromised mice. Our data suggests that chemotherapy is not effective in killing these cells $CD44^{+}/CD24^{-/low}$ cells, with ability to form new cancers. By isolating these $CD44^{+}/CD24^{-/low}$ cells and using high-throughput genomic profiling, we have determined, in the largest data set to date, that certain pathways like the Notch, EGFR/PI3K, and Hedgehog pathways, may be important in cancer stem cells. In keeping with these findings, we conducted a clinical trial with lapatinib, an EGFR/HER2 inhibitor, that showed for the first time, that “cancer stem cells” could be targeted, with a decrease in both $CD44^{+}/CD24^{-/low}$ and mammospheres. These exciting results have been reported in the *Journal of National Cancer Institute.*

Intrinsic chemoresistance of cancer stem cells

Current systemic therapies including endocrine therapy are initially effective in killing breast cancer cells and controlling tumor growth. Yet, nearly all patients with metastatic disease and a quarter of those with early disease relapse over time despite initial response. In part, this may be due to existing therapies which are aimed only at proliferative and apoptotic pathways resulting in temporary therapy-induced shrinkage of human breast cancers. Recent evidence supports the existence of cancer stem cells, which are characterized by their relative quiescence but a capacity to self-renew and divide indefinitely. A unique component of these studies is the availability of human biopsy samples obtained before and after therapy in breast cancer patients receiving preoperative (neoadjuvant) endocrine therapy.

Objective/Hypothesis: We hypothesize that breast cancer stem cells are resistant to chemotherapy, and that inhibition of stem cell self-renewal pathways will improve breast cancer patient outcome.

Specific Aims: To determine whether breast cancer biopsies obtained from patients enrolled in preoperative clinical trials are enriched for cancer stem cells after neoadjuvant chemotherapy

Results: We report that tumorigenic breast cancer cells were intrinsically chemoresistant – chemotherapy led to increased $CD44^{+}/CD24^{-/low}$ cells, increased self-renewal capacity on mammosphere formation efficiency (MSFE) assays. In matched human breast cancer biopsies (n=31 pairs) the relative proportion of $CD44^{+}/CD24^{-/low}$ cells increased with chemotherapy from a baseline mean of 4.7% to 13.6% after 12 weeks of chemotherapy ($p<0.0001$) (Fig. 1). Consistent with the increase in the relative proportion of tumorigenic cells, mean MSFE was significantly increased after chemotherapy in matched pre- and post-chemotherapy samples ($p<0.0001$) (Fig. 2). Conversely, in patients with HER2 overexpressing tumors, lapatinib (EGFR/HER2 tyrosine kinase inhibitor) did not increase tumorigenic cells, but led to a statistically non-significant decrease in matched biopsies from a baseline mean of 10.6% to 4.7% after 6 weeks of lapatinib (Fig. 3). Consistent with its effect on tumorigenic cells, lapatinib treatment again did not show an increase as with chemotherapy, but led to a non-significant decrease in self-renewal capacity as measured by MSFE (Fig. 3). Conclusion These studies provide the first clinical evidence for a subpopulation of chemotherapy-resistant breast cancer-initiating cells, and suggest that specific pathway inhibitors in combination with conventional chemotherapy may provide a therapeutic strategy for eliminating these cells in order to decrease recurrence and improve long-term survival.

Publications:

Intrinsic Resistance of Tumorigenic Breast Cancer Cells to Chemotherapy. X Li, M Lewis, C Creighton, H Wong, X Zhang, H Pham, T Gray, MC Gutierrez, CK Osborne, M Wu, S Hilsenbeck, G Chamness, J Rosen and **JC Chang.** *Journal of National Cancer Institute* 2008 (in press).

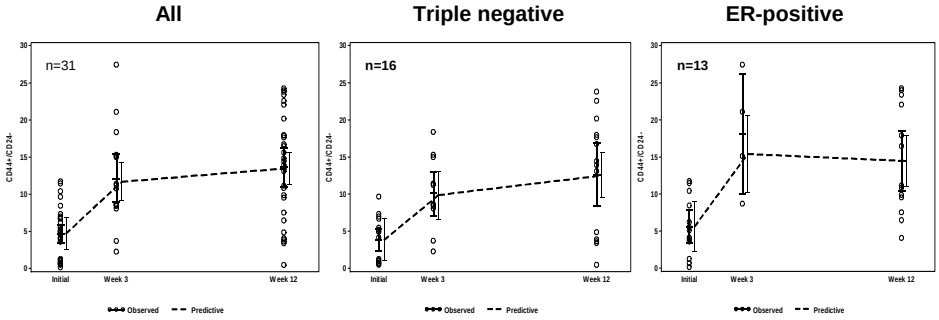

Fig 1. Effect of chemotherapy on mean % CD44+/CD24- cells before, during, and after treatment. Predictive values and their standard errors were estimated by linear mixed-effected models. Error bars represent two standard errors of the mean of experiments at baseline and each time point of follow-up.

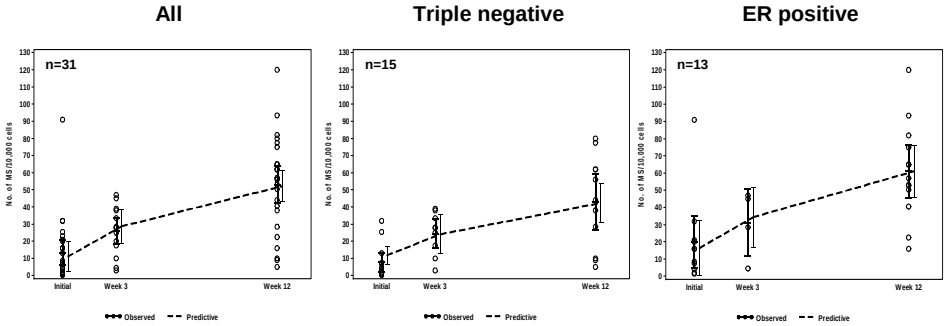

Fig 2 . Similar effect of chemotherapy on mean MSFE before, during, and after treatment in:
A). All patients
B). Triple negative patients
C). ER-positive patients

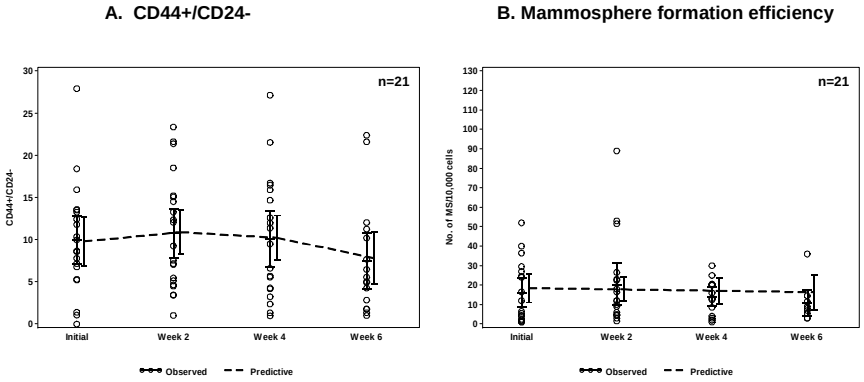

Fig 3. Effect of lapatinib on mean % CD44+/CD24- cells and MSFE before, during, and after treatment. Predictive values and their standard errors were estimated by linear mixed-effected models. Error bars represent two standard errors of the mean of experiments at baseline and each time point of follow-up.
A). Unlike chemotherapy, tumorigenic cells did not increase with lapatinib treatment, and showed a non-significant decrease.
B). With lapatinib, MSFE showed a non-significant decrease from initial to week 6.

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REPORT OF INVENTIONS AND SUBCONTRACTS

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13. ABSTRACT (Maximum 200 words)

Statement of Work Task 1: Development of/Preparation for Metastatic Chemotherapy Parent Protocol
6 sites (4 US and 1 Peru) open under the Master Protocol dated 20MAY2005.

2 additional US sites are in the regulatory process for opening under the Protocol Amendment 12JAN2007. This amendment will allow patients to enroll to the trial without obtaining fresh frozen tissue as long as a formalin-fixed paraffin embedded tissue block is available for analysis.

All sites will submit this amendment to assist in patient enrollment on this trial. Of the 5 current open sites, only one has the amended protocol approved and is awaiting the Department of Defense's approval for enrollment of patients. All remaining sites are in the process of obtaining approval of the amended protocol.

2 sites closed to accrual due to lack of accrual at their site.

Monthly teleconferences conducted with George Sledge, M.D and Cores. Face-to-Face meetings conducted 02JUN2007 and 20MAR2007.

3 sites (all US) are open under the second protocol dated 12JAN2007.

2 additional sites (US and Peru) are in the regulatory process for opening the second trial.

There is a Retrospective / Prospective Research Plan currently collecting data and formalin-fixed paraffin embedded tissue samples. 64 samples have been retrieved to date.

Statement of Work Task 2: Performance of Metastatic Chemotherapy Trial/Tissue Collection/Patient Follow-up
Forty patients enrolled to the trial in the US and Peru. Tissue collection and submission to central laboratory conducted as planned.

Statement of Work Task 3: Analysis of Tissues by Laboratory Cores
Five patients enrolled to the trial in the US. Tissue collection and submission to central laboratory conducted as planned.

Statement of Work Task 4: Performance of Prospective Validation Trial
Awaiting completion of Task 1-3

Statement of Work Task 5: Performance of Investigational Agent Trials
The third protocol draft and informed consent draft has been changed to be a tissue collection protocol only. Expected start of this trial is October 2008.

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Introduction

The primary objective of the pharmacodynamics/pharmacogenomics core is to develop user-friendly techniques that are readily available to the clinician for measuring a specific aspect of response and/or toxicity, which will lead to the individualization of therapy. Critical determinants that govern individual responsiveness will be identified as specific "signatures". One important advantage of measuring these signatures is that they will be directly compared to and contrasted with the genomic and proteomic analyses.

We identified several determinants of capecitabine response and toxicity that are now being evaluated in formalin-fixed paraffin-embedded (FFPE) archival samples from COE-05 and we will soon begin analysis of fresh frozen tissue samples from COE-01. Techniques to evaluate markers for gemcitabine have been developed and will be performed as soon as the samples from COE-01 and COE-05 become available. In both capecitabine and gemcitabine arms, patterns of RNA expression will be explored using c**D**NA-mediated **A**nnealing, **S**election, extension and **L**igation (DASL) on a customized panel of over 500 breast cancer-related genes. This novel technique yields accurate and consistent results even when using degraded RNA such as RNA isolated from FFPE samples.

Fluoropyrimidine nucleosides analogues, with 5-fluorouracil (5-FU), capecitabine and gemcitabine as principal representatives of this class of chemotherapeutic agents, have been the standard treatment for a wide range of common solid tumors including breast cancer. Unfortunately, we still lack reliable methods for the selection of patients who will have the best chance to benefit from capecitabine or gemcitabine-based treatments.

Capecitabine:

Molecular pharmacology and mechanism of action

Attempts to increase the efficacy and tolerability of fluoropyrimidine treatment have led to the development of capecitabine (Xeloda™), a prodrug transformed into 5-FU preferentially in tumors (Figure 1). Capecitabine is activated at the tumor site by the enzyme thymidine phosphorylase (TP) (Miwa et al., 1998), taking advantage of the fact this enzyme is more highly expressed in tumor tissue (Takebayashi et al., 1996), including breast cancer (Kobayashi et al., 2005). Capecitabine and its intermediate metabolite, 5'-deoxy-5-fluorouridine (5'-DFUR), are not cytotoxic but become effective only after conversion to 5-fluorouracil (5-FU) by TP and further transformations into fluorodeoxyuridine monophosphate (FdUMP) and, fluorouridine triphosphate (FUTP) (Miwa et al., 1998). Inhibition of the enzyme thymidylate synthase (TS) by FdUMP is considered to be the main mechanism of action of fluoropyrimidine treatments, including capecitabine (Walko and Lindley, 2005) (**Figure 1**).

TS is an important enzyme in pyrimidine metabolism which is crucial for *de novo* thymidine nucleotide synthesis used for DNA replication and cellular division (Peters et al., 1995). Inhibition of TS occurs as a result of the formation of an inactive ternary covalent complex between TS, FdUMP and 5-10 methylenetetrahydrofolate (CH2FH4). The stability of this ternary complex is highly dependent on the availability of CH2FH4 or one of its polylglutamates (Houghton et al., 1982; Houghton and Houghton, 1983). Dihydrofolate reductase (DHFR) is a key enzyme involved in folate metabolism and plays a role in the *de novo* pathway of pyrimidine biosynthesis that has been linked to the modulation of fluoropyrimidine treatments (Capiaux et al., 2003; Will and Dolnick, 1989).

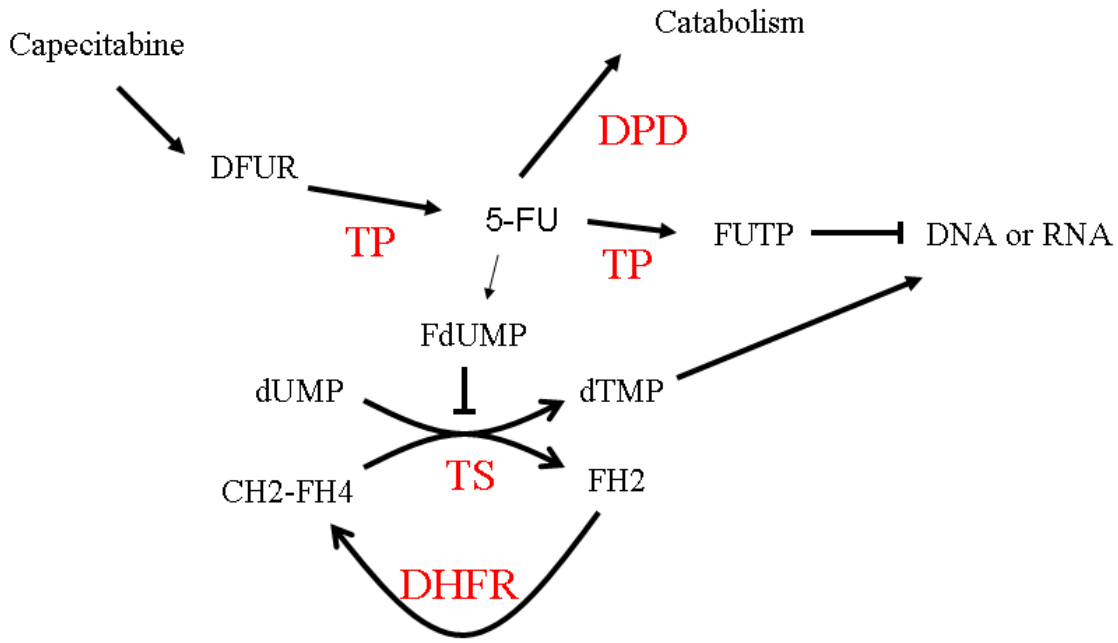


Figure 1: Pathway of capecitabine metabolism and catabolism. Abbreviations: CH2-FH4, 5-10 methylenetetrahydrofolate; DFUR, 5'-deoxy-5-fluorouridine; DHFR, dihydrofolate reductase (GeneID: 1719; EC 1.5.1.3); DPD, dihydropyrimidine dehydrogenase (GeneID: 1806; EC 1.3.1.2); dTMP, deoxythymidine-5'-monophosphate; dUMP, deoxyuridine-5'-monophosphate; FdUMP, 5-flurodeoxyuridine-5'-monophosphate; FH2, dihydrofolate; 5-FU, 5-fluorouracil; FUTP, Fluorouridine triphosphate; TP, Thymidine phosphorylase (GeneID: 1890; EC 2.4.2.4); TS, Thymidylate synthase (GeneID: 7298; EC 2.1.1.45).

Dehydropyrimidine dehydrogenase (DPD) is the enzyme responsible for the first and rate limiting step in the catabolic conversion of 5-FU to inactive metabolites and decreases 5-FU levels within cells (Johnson et al., 1997; Lee et al., 2004; Lu et al., 1993). Several studies have underlined the role of DPD deficiency in the development of severe 5-FU toxicity and conversely DPD overexpression is associated with resistance to these therapies (Kornmann et al., 2003). Both elevated DPD gene copy number and mRNA expression were linked to increased resistance to capecitabine and other 5-FU-based

treatments in several human cells lines, including breast (Kobunai et al., 2007).

Since DPD is rate limiting for the catabolic pathway and TP is key to the production of active capecitabine metabolites, the TP/DPD ratio has been frequently used to correlate with capecitabine or 5-FU efficacy. It was first shown that a high TP to DPD ratio correlated with a high capecitabine efficacy and conversely a low TP/DPD ratio was linked to resistance in a large number of xenograft models, including breast (Ishikawa et al., 1998). Recent immunohistochemical (IHC) data has shown that a higher TP/DPD ratio correlates with a better clinical response in a small cohort of breast cancer patients treated with capecitabine monotherapy (Honda et al., 2008).

Similarly, RT-PCR analysis of tumors from 22 breast cancer patients revealed that the group of patients expressing high levels of TS and DPD were resistant to 5-FU, whereas those patients expressing low levels of TS and DPD were sensitive to 5-FU (Kakimoto et al., 2005)

Using IHC, it was shown that high levels of TP expression in tumors was a significant prognostic indicator of 5'-DFUR efficacy in breast cancer patients (Tominaga et al., 2002).

Therefore, the fluoropyrimidine pathway enzymes, TP, TS, DPD and DHFR, are potential candidate biomarkers that could be used to predict tumor response to capecitabine. Efforts have been made to select assays that would be easily accessible to clinicians in order to correlate protein/enzyme expression profiles with disease state, therapy and drug response. This work will provide invaluable insight into monitoring inter-individual variations in

efficacy and toxicity of capecitabine and these observations could be used to help select appropriate drug and dosage regimens for each patient.

Evaluation of markers for capecitabine

In order to identify pharmacokinetic signatures for capecitabine, FISH assays were established for the detection of amplification or deletion of genes for TS (gene symbol TYMS), TP (gene symbol TYMP), and DHFR (gene symbol DHFR) as well as qPCR mRNA expression assays for TS, TP, DPD and DHFR.

For investigation of TS, DHFR and TP gene copy number, newly developed FISH probes (Dako, Glostrup, Denmark) have been used on 5µm FFPE tissue slices. Hybridization signals have been evaluated using the ratio of red signals for TS, DHFR or TP to green signals for a reference sequence on the same chromosome in at least 60 morphologically intact and non-overlapping nuclei. Tumors have been classified as TS, DHFR or TP amplified (TS/REF, DHFR/REF or TP/REF ≥ 2.0), or deleted (TS/REF, DHFR/REF or TP /REF ≤0.8). Gene copy number alterations for TS, DHFR and TP in a first set of 14 patients are shown in **Table 1**.

The small number of patients in this initial set did not yield any significant correlation between gene copy alterations for these three genes and the available clinical data. Some preliminary data using the same FISH probes on specimens from 24 breast cancer patients treated with capecitabine has been presented in abstract form (Christensen et al., 2006). This small study revealed that in the 9 patients that had a time to progression (TTP) of less than 3 months, all but 2 had copy number alterations in at least one of these

three genes, and that no such alterations were observed in the 15 patients who had a clinical benefit from capecitabine therapy (TTP >6 months).

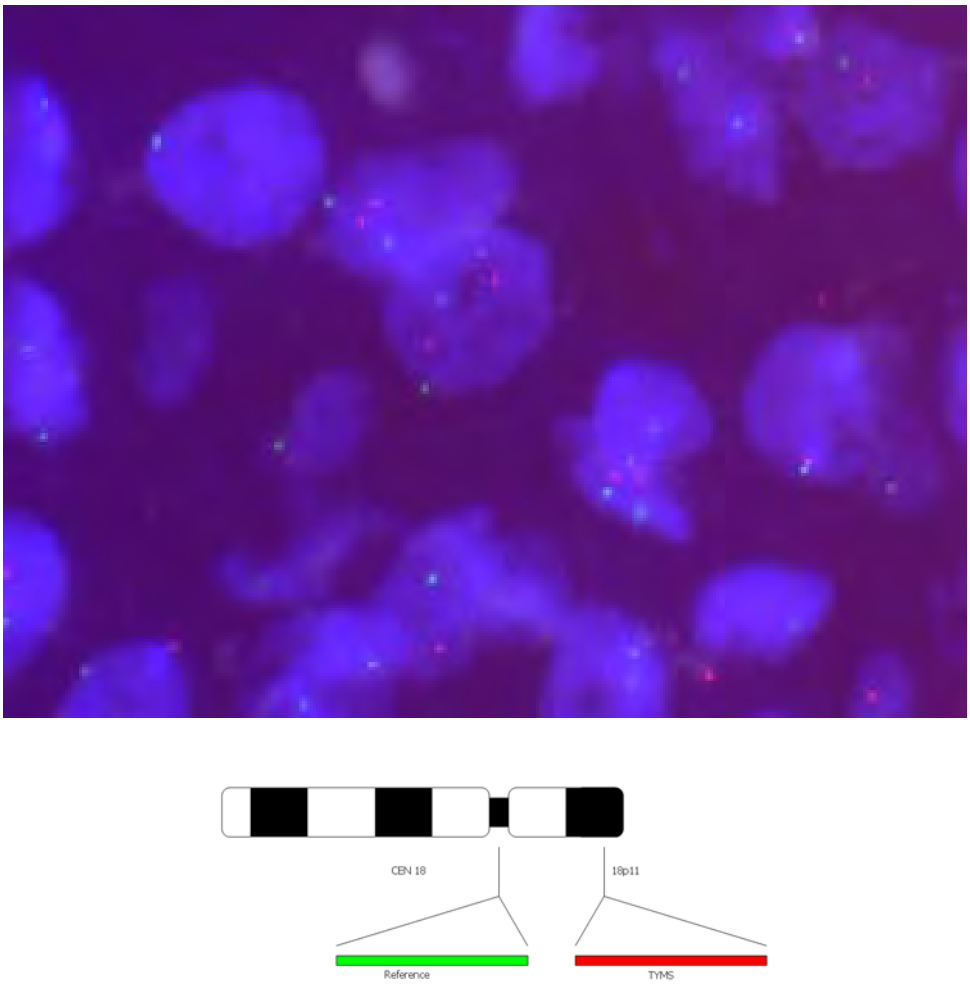


Figure 2: Representative photograph of cells with a TYMS gene deletion, with reference signals for the centromere of chromosome 18 (green dots) and signals for TYMS (red dots) in nuclei (blue). A ratio of red/green signals (TYMS/CEN18) equal to or above 2 was considered as amplified and a ratio below 0.8 was considered to be deleted. It should be noted that only one plane of focus is shown; whereas the scoring of green and red dots used to calculate the ratio was performed in all available focus planes and in 60 morphologically intact and non-overlapping nuclei. 100X magnification.

Table 1: Gene copy number alterations for TS, DHFR and TP in 14 breast cancer patients' specimens assessed using FISH. Samples with ratios of less than 0.8 were considered as having a gene deletion (green). No cases of gene amplification were noted in these samples.

Patient	TS /REF ratio	DHFR /REF ratio	TP /REF ratio
COE-05-0002	1.15	1.05	1.15
COE-05-0006	0.92		1.01
COE-05-0008	0.64	1.12	0.95
COE-05-0010	0.78	0.98	1.05
COE-05-0012	1.03		1.03
COE-05-0014	1.17		0.93
COE-05-0017	1.20	0.88	0.98
COE-05-0019	1.08		0.89
COE-05-0020	1.04	0.94	1.16
COE-05-0024	1.10	1.26	1.10
COE-05-0029	0.95		0.93
COE-05-0030	1.09		1.15
COE-05-0035	0.90	0.92	0.93
COE-05-0036	0.74	1.08	0.79

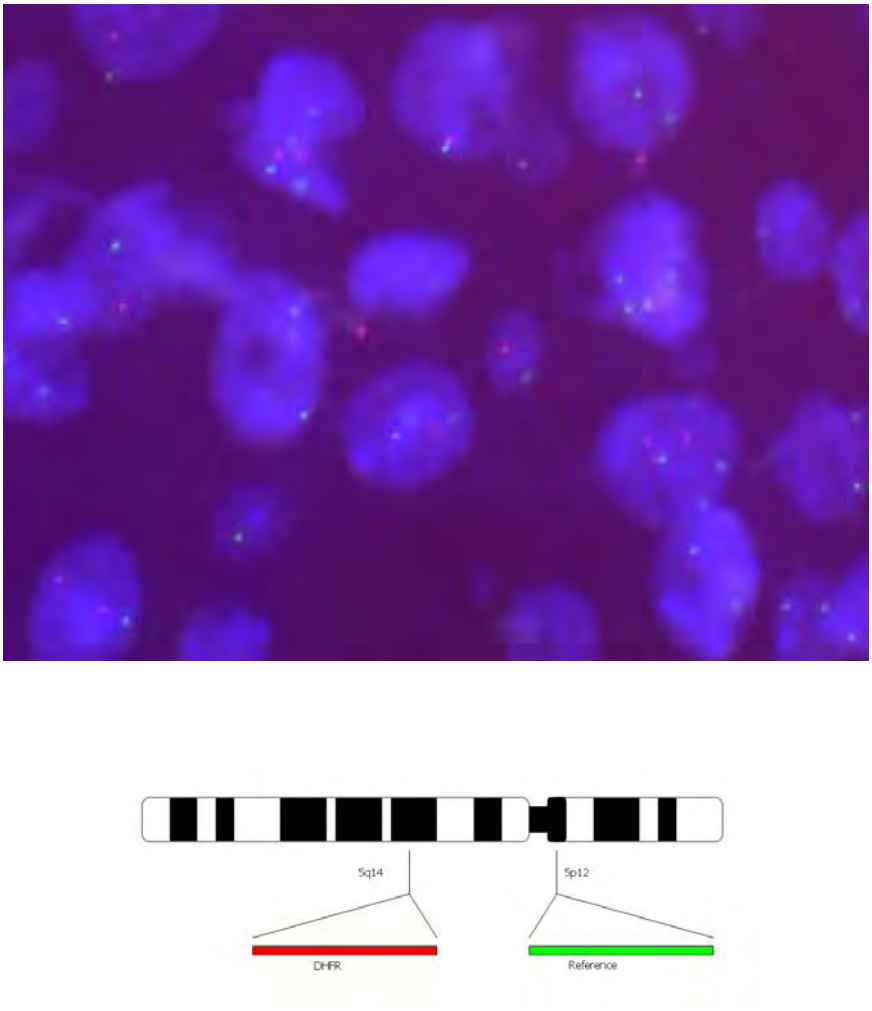


Figure 3: Representative photograph of cells with normal DHFR gene, with reference signals for chromosome 5 (green dots) and signals for DHFR (red dots) in nuclei (blue). A ratio of red/green signals (DHFR/REF) equal or above 2 was considered as amplified and a ratio below 0.8 was considered to be deleted. It should be noted that only one plane of focus is shown; whereas the scoring of green and red dots used to calculate the ratio was performed in all available focus planes and in 60 morphologically intact and non-overlapping nuclei. 100X magnification.

Analysis of samples currently being collected from a larger cohort of patients will certainly help in identifying critical determinants of capecitabine efficacy and toxicity. Identification of HER2 as a marker of response to Herceptin has led to the wide use of HER2 FISH probes to identify patients who will most likely benefit from such treatment (Pegram et al., 1998; Slamon et al., 2001).

Currently, FISH assays are readily available to clinicians and the identification of markers able to help predict response to fluoropyrimidine treatment would represent a major advance for patient care

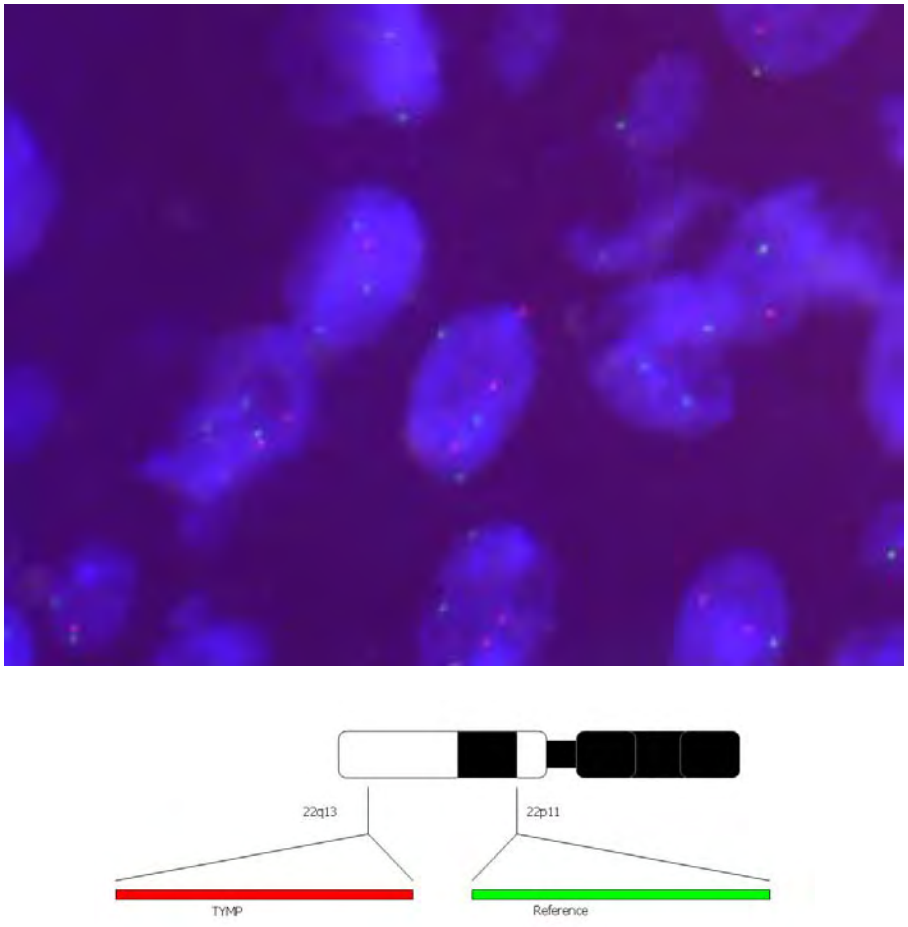


Figure 4: Representative photograph of cells with normal TYMP gene, with reference signals for chromosome 5 (green dots) and signals for TYMP (red dots) in nuclei (blue). A ratio of red/green signals (TYMP/REF) equal or above 2 was considered as amplified and a ratio below 0.8 was considered to be deleted. It should be noted that only one plane of focus is shown; whereas the scoring of green and red dots used to calculate the ratio was performed in all available focus planes and in 60 morphologically intact and non-overlapping nuclei. 100X magnification.

Initial results for RNA extraction from archival formalin-fixed paraffin-embedded (FFPE) samples

We isolated total RNA from five to six 5μm thick FFPE sections containing at least 50% tumor cells using the Ambion RecoverAll RNA Kit from each of 47 FFPE breast tumor samples coming from 37 patients treated with capecitabine using our optimized protocol specifically designed for isolation of RNA from FFPE specimens (Abramovitz et al., 2008). Total RNA yields ranged from 0.14 to 57.6 μg and, in the vast majority of cases, at least 1 μg of total RNA was obtained with an average yield greater than 8 μg. RNA concentration ranged from 5 to 1310 ng/μl with an average of 218 ng/μl and the concentrations paralleled the total yield (**Figure 5**).

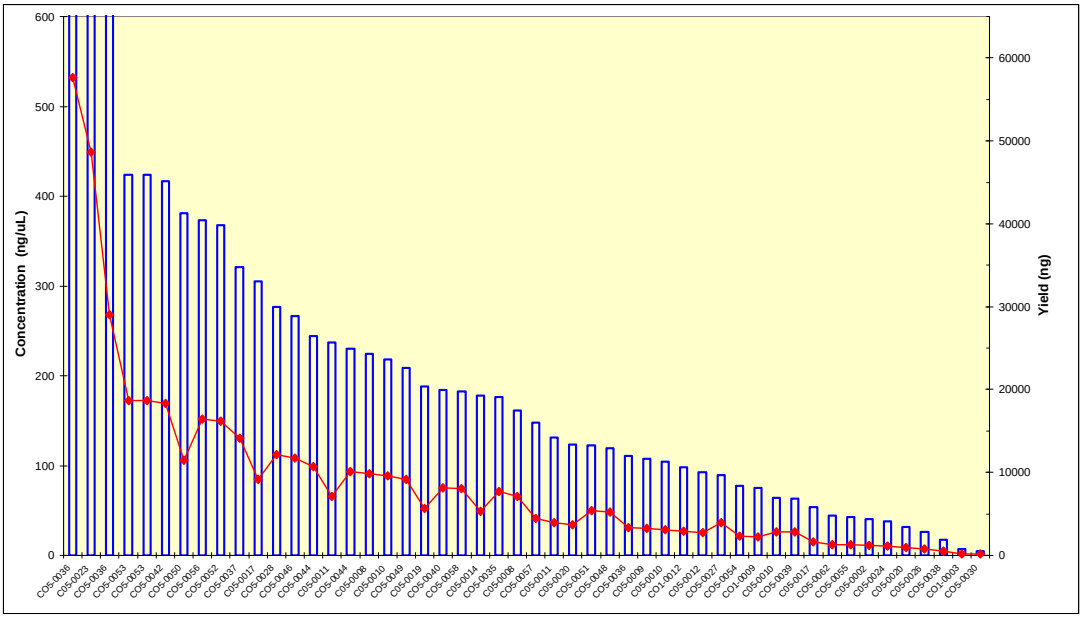


Figure 5: Concentration and total yield for RNA extracted in 47 FFPE breast tumor samples from 37 patients treated with capecitabine. Blue bars represent RNA concentration for each sample in μg/μl and red line represents yield in ng.

To test the quality of the RNA extracted from the FFPE samples, the classical 260/280 absorbance ratio was measured using Nanodrop™ technology. This 260/280 absorbance ratio was consistently near a value of 2 with an average of 2.03, which is considered excellent and demonstrates there is little if any contamination in our samples (data not shown).

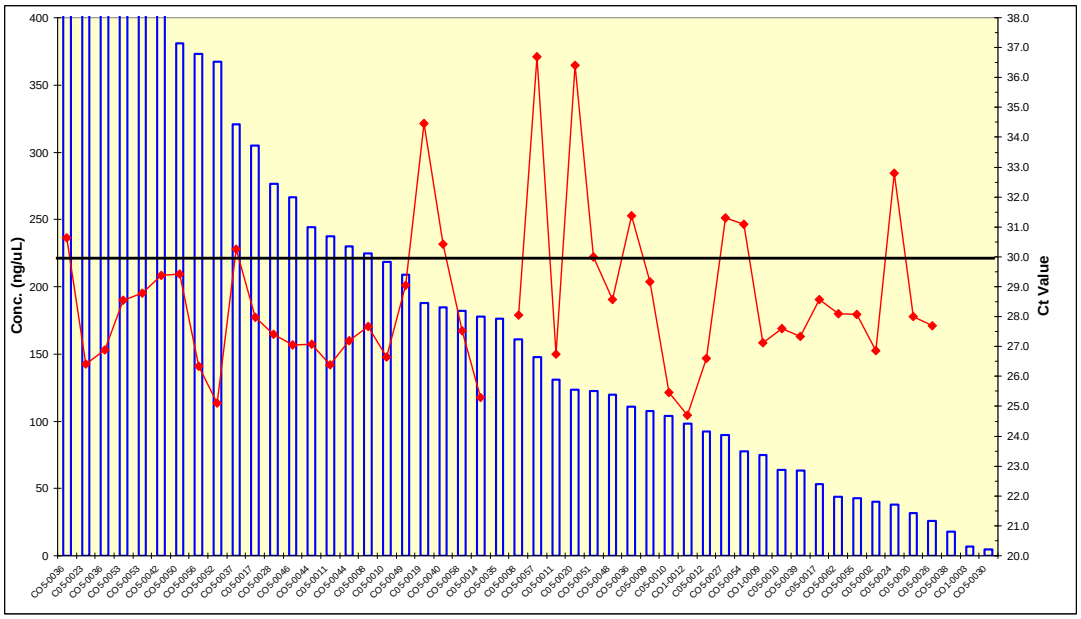


Figure 6: Concentration and quality control for RNA extracted from 47 FFPE breast tumor samples from 37 patients treated with capecitabine. Blue bars represent RNA concentration for each sample in μg/μl and red line represents Ct values for the amplification of the housekeeping gene RPL13a. The black line was set at a Ct value of 29 which is the upper limit usually used to consider RNA of sufficient quality for use.

As a second and more refined method to evaluate RNA integrity, we used real-time quantitative polymerase chain reaction (qPCR) performed on a 90bp fragment of the highly abundant transcript of the ribosomal protein L13a (RPL13a). When performing real-time qPCR, the fluorescent signal increases exponentially in direct relation with the cDNA copy number present

initially and subsequently will cross a determined threshold. The number of PCR cycles required for the fluorescent signal to cross this threshold is called the Ct value. For a particular gene product, a large number of cDNA copies will yield a low Ct value and conversely for a lowly expressed gene. When qPCR is performed on RNA prepared from FFPE sections using the RPL13a primer set, the Ct value that would reflect a good quality sample should be ≤ 29.

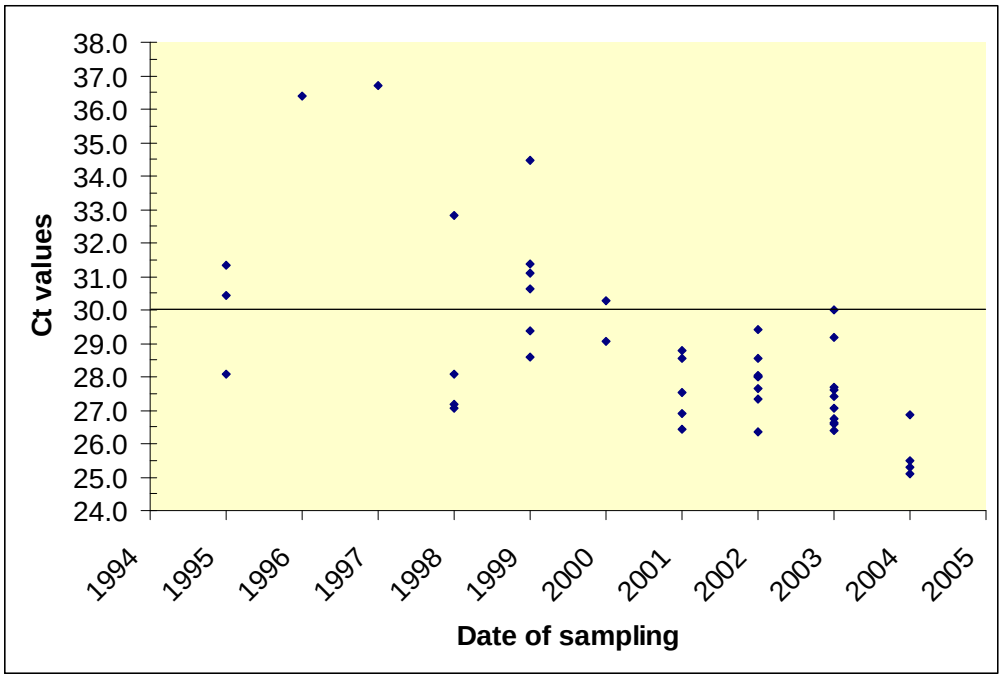


Figure 7: Relation between Ct values and date of sampling for RNA extracted from 47 FFPE breast tumor samples from 37 patients treated with capecitabine. Ct values are for the amplification of the reference gene RPL13a. The black line was set at a Ct value of 29 which is the upper limit usually used to consider RNA of sufficient quality for use.

Of the 47 samples evaluated 11 (23%) had Ct values for RPL13a above 29 and 3 (6%) had insufficient amounts of RNA to perform qPCR amplification

(**Figure 6**). The concentration of extracted RNA does not seem to correlate with their Ct value, suggesting the integrity of the RNA has little relation with the amount or concentration extracted (**Figure 6**). Interestingly, we found higher Ct values in many samples more than eight years old suggesting the RNA in some of these older FFPE samples has been degraded to the point where it might compromise its use for PCR-based techniques (**Figure 7**). This effect of sample age on Ct value is apparent when looking more closely at specimens from four patients for which samples collected at different time points were available. In all four cases, older samples had higher Ct values and only samples more than 10 years old had Ct values higher than 29 (**Figure 8**).

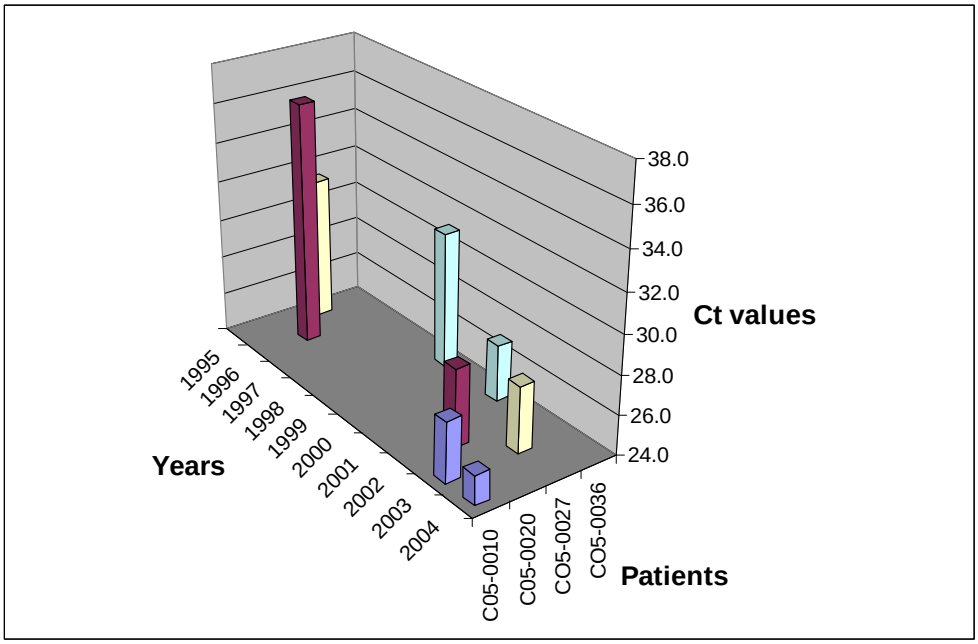


Figure 8: Relation between Ct value and date of sampling for RNA from 4 patients treated with capecitabine for which samples were taken at different times. Higher Ct values are observed for the older samples compared to the more recent ones for every patient and these were above 29 for all samples more than 8 years old.

In conclusion, these results demonstrate that RNA extracted from archival FFPE specimens can be used for PCR based techniques and that some unusable samples should be expected when using older material emphasizing the need for quality control on each and every case.

Gemcitabine

Molecular pharmacology and mechanisms of action

The pyrimidine nucleoside analogue gemcitabine (Gemzar™) is frequently used in the treatment of breast cancer patients with solid tumors (Silvestris et al., 2007). Gemcitabine (2',2'-difluorodeoxycytidine [dFdC]) is a cell cycle-dependent (S-phase-specific) deoxycytidine analogue and must first be transported into the cell and phosphorylated to its active, triphosphate form (Bergman et al., 2002).

Activation of gemcitabine requires phosphorylation to mono-, di-, and triphosphates and the first crucial step is the phosphorylation catalyzed by deoxycytidine kinase (dCK) (**Figure 9**) (Bergman et al., 2002). Difluorodeoxycytidine triphosphate (dFdCTP), the main active metabolite of gemcitabine, competes with deoxycytidine triphosphate for incorporation into DNA (Veltkamp et al., 2008). In addition, gemcitabine diphosphate (dFdCDP) inhibits ribonucleotide reductase M1 (RRM1) (Heinemann et al., 1990), thereby depleting dCTP pools and facilitating incorporation of dFdCTP into DNA.

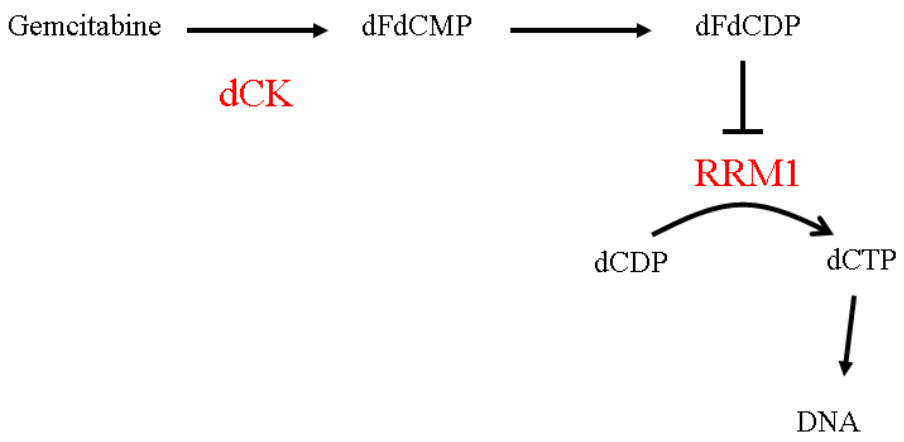


Figure 9: Pathway of gemcitabine metabolism and mechanism of action. Abbreviations dCDP, deoxycytidine diphosphate; dCK, deoxycytidine kinase; dFdCMP, difluorodeoxycytidine monophosphate; dFdCDP, difluorodeoxycytidine diphosphate; dCTP, deoxycytidine triphosphate. RRMI, ribonucleotide reductase M1.

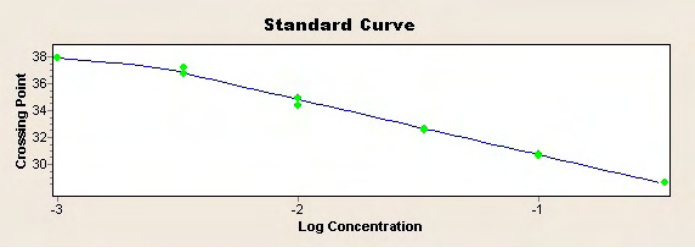
Phosphorylation of gemcitabine to dFdCMP by dCK is the rate limiting step in the formation of its metabolite dFdCTP and the activity of dCK is recognized as an important factor in the overall toxicity of gemcitabine (Yardley, 2004). Xenograft models in nude mice have shown that cDK expression is positively correlated with enhanced intracellular dFdCTP accumulation and with the anti-tumor activity of gemcitabine (Blackstock et al., 2001).

Ribonucleotide reductase M1 (RRM1) is one of gemcitabine's targets and is a logical candidate as a biomarker, along with members of specialized transport systems required for the passage of nucleoside analogs that could be used to predict tumor response to gemcitabine. RRM1 expression was shown to be a determinant of the efficacy of gemcitabine in lymphocytic B-leukemia patients (Giovannetti et al., 2007).

Evaluation of markers for gemcitabine

In an attempt to elucidate the role of dCK and RRM1 expression in the efficacy and toxicity of gemcitabine in breast cancer, qPCR assays were developed to measure the expression of these genes in FFPE and fresh frozen samples. A validation set of 5 FFPE samples was used to evaluate the feasibility of our approach. **Figure 10** and **11** show representative calibration and amplification curves for dCK and RRM1, respectively.

A



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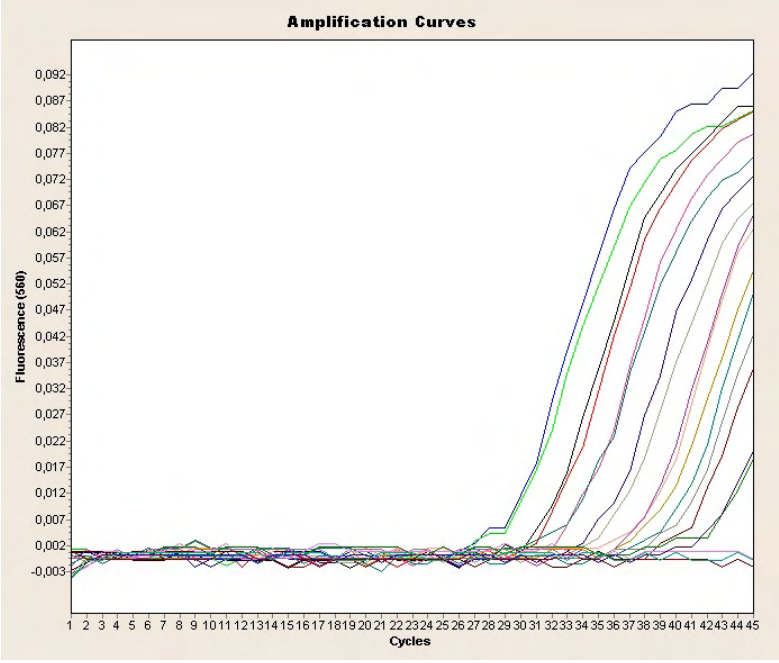
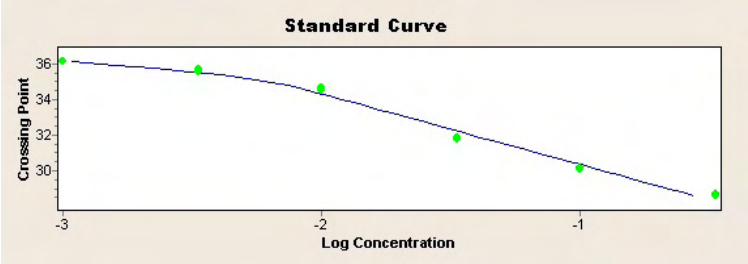


Figure 10: Validation of the qPCR analysis of deoxycytidine kinase (dCK) **A)** Calibration curve showing the linearity of the reaction over several orders of magnitude. **B)** Representative amplification curves for dCK.

A



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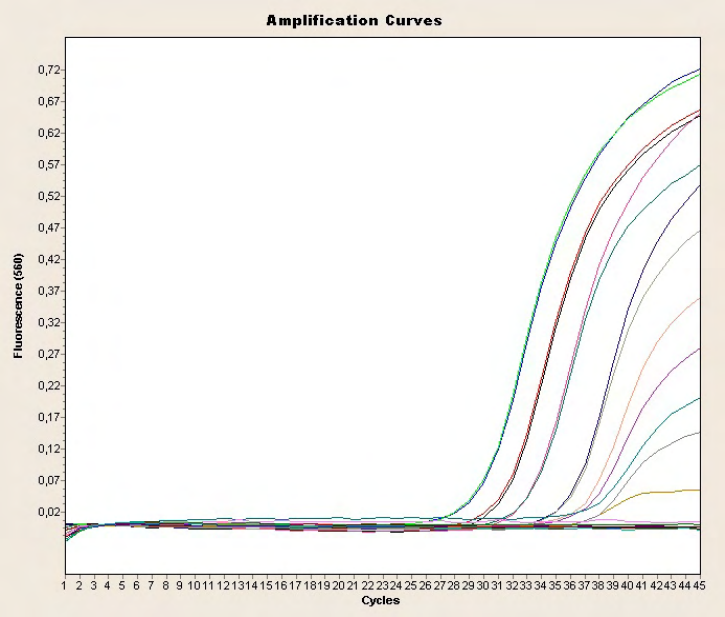

Figure 11: Validation of the qPCR analysis of ribonucleotide reductase M1 (RRM1) **A)** Calibration curve showing the linearity of the reaction over several orders of magnitude. **B)** Representative amplification curves for RRM1.

In summary, assays to evaluate dCK and RRM1 expression in FFPE as well as fresh frozen tissues were validated. The above assays will be compared and correlated with data generated using the DASL assay.

Expression profiling using our custom 512-gene Breast Cancer Panel

Custom 512-gene breast cancer panel

Our custom panel of breast-cancer related genes will be used in the DASL assay to analyze RNA derived from FFPE tumor tissue samples. Many of the genes selected for this custom breast cancer panel have previously been identified as signature genes, both prognostic and predictive, from various breast cancer microarray studies. Other selected genes have been implicated as playing a role in a number of cancer-related processes including angiogenesis and metastasis, or as potential markers in breast cancer. The custom panel is made up of 512 genes and includes genes selected from, but not limited to, studies shown below:

- Breast cancer subtype-selective genes (van't Veer et al., 2002; Yehiely et al., 2006)
- 70-gene prognostic signature for patients with node negative breast cancer (van de Vijver et al., 2002)
- 44-gene predictive signature for patients treated with tamoxifen (Jansen et al., 2005)
- 64-gene prognostic signature for predicting patients with good and poor outcomes to therapy (Pawitan et al., 2005)
- 92-gene predictive signature for patients treated with docetaxel (Chang et al., 2005)
- 74-gene predictive signature of complete pathologic response to neoadjuvant chemotherapy (Ayers et al., 2004)
- OncotypeDX's 16 cancer-related genes (Paik et al., 2004)
- 32-gene expression signature that distinguishes p53-mutant and wild-type breast cancer tumors (Miller et al., 2005)

- Breast cancer bone and lung metastatic-related genes (Kang et al., 2003; Minn et al., 2005). Recently identified cancer-related genes (Gonzalez et al., 2003; Heuze-Vourc'h et al., 2005; McLean et al., 2005; Zhao et al., 2003)
- Angiogenesis-related genes (Heuze-Vourc'h et al., 2005; Khatua et al., 2003; Zhong et al., 2000)
- Thrombosis-related genes (Kwaan et al., 2003)
- Other cancer-related genes (oncogenes, cell cycle genes, proliferation genes, telomerase-related genes, breast cancer stem cell genes, senescence-related genes, apoptosis-related genes, DNA repair genes, etc.) (Brabletz et al., 2005; Collado et al., 2005; Dikmen et al., 2005)

Validation of the DASL assay using the custom 512-gene breast cancer panel

To validate both the DASL assay and our custom Breast cancer panel, we performed the DASL assay on 187 samples from 87 patients in the six major breast cancer subtypes. Data from DASL experiments were scanned and interpreted using Illumina's BeadStudio software. Prior to analysis, samples which failed (criteria being a detection p-value < 0.05 in less than 40% of the samples) were removed from the data sets. Reference RNA, and samples with no IHC data (i.e. ER, ESR1; PR, PGR; HER2, ERBB2 status) were also removed. Therefore, with removal of these samples from further analysis, we performed DASL analysis on 175 samples from 87 patients in six major breast cancer subtypes. Non-normalized signal intensity data was exported from BeadStudio and analyzed for correlations in Excel and Access.

To perform initial validation of the custom panel across all genes, technical replicates within the custom cancer panel Sentrix Array Matrices (SAMs) were correlated,

first within (intra-SAM) and then between (inter- SAM) SAM arrays. Technical replicates, both intra- and inter-SAM, yielded excellent correlations, R^2 coefficients of 0.972 and 0.948, respectively (**Figure 12)**.

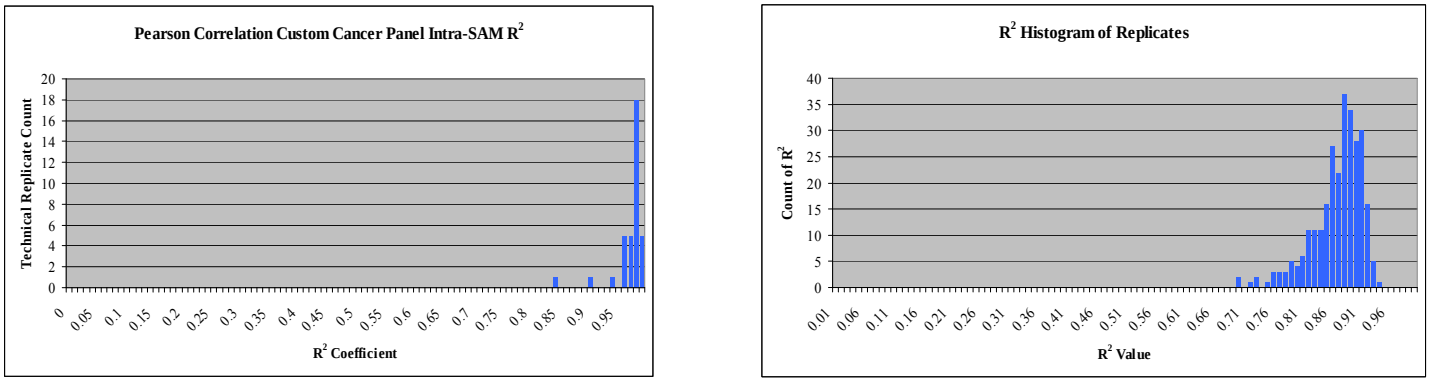

Figure 12. Correlation of technical replicates within (intra-SAM) and between (inter-SAM) SAM arrays in the DASL assay using the custom panel.

To confirm experimental validity of our custom breast cancer DASL panel, technical replicates were placed on two custom breast cancer panel SAMs and two standard cancer panel (Illumina's 502-gene cancer panel) SAMs.

For correlation between cancer panels, data was taken from SAMs 1892661004 & 1892661022 for the standard cancer panel and SAMs 1842787020 & 1892661005 for the custom breast cancer panel. In common between these cancer panels is a set of 152 genes (see appendix A for list of genes). In all there were 277 pair-wise correlations between panels, yielding an average R^2 value of 0.877. This indicates a high reproducibility for DASL experiments between at least 152 probed genes in the standard and custom breast cancer panels. Correlation of technical replicates between custom and

standard cancer panels within these 152 common genes confirmed assay quality in the custom cancer panel (**Figure 13).** These data together with the data for correlation of technical replicates within the custom cancer panel across all 512 genes, indicates a high level of reproducibility for FFPE tissue experiments using our custom breast cancer panel.

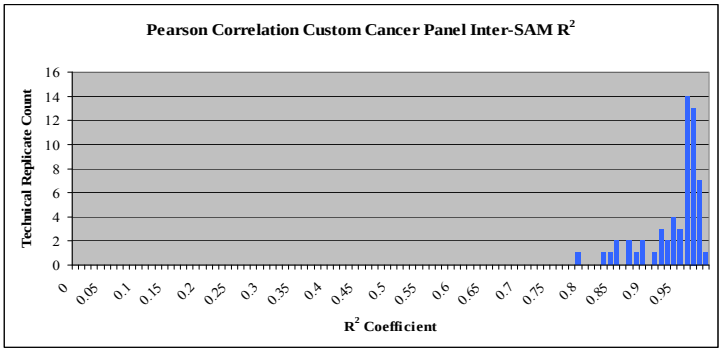

Figure 13. Correlation between 152 common genes on Illumina standard cancer panel and custom breast cancer panel. Histogram of R^2 correlation of probes between the custom and standard cancer panels is shown.

In addition, we plotted DASL assay data for expression of ESR1, PGR, and ERBB2 receptors according to receptor subtype as determined by IHC (i.e. ESR1+PGR+ERBB2+, ESR1+PGR-ERBB2+, ESR1-PGR-ERBB2-, ESR1+PGR-ERBB2-, ESR1+PGR+ERBB2-, ESR1-PGR-ERBB2+) on these 87 patients and found excellent correlation between DASL data and IHC data (**Figure 14**). Thus, results are concordant and further confirm the use of the custom panel in the DASL assay.

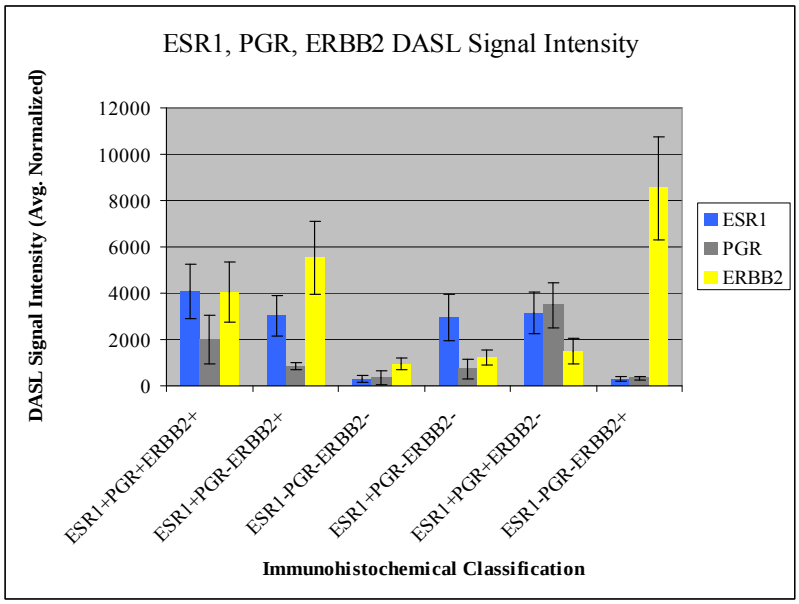

Figure 14. DASL assay data for ESR1, PGR and ERBB2. ESR1, PGR and ERBB2 expression levels in the DASL assay shows excellent correlation with receptor expression as determined by IHC.

To validate the relevance of the genes selected for our custom cancer panel, we conducted an unsupervised hierarchical clustering based on expression correlation. Expression values were quantile normalized with plate scaling to adjust for intensity differences between Sentrix arrays. The following heatmap (**Figure 15**) illustrates the clustering of breast cancer IHC defined subtypes and further authenticates the relevance of the genes selected for the custom cancer panel in that clinically types are organized together such as the TNT (ESR1- PGR- ERBB2-) subtype which clusters to the left side of **Figure 15**.

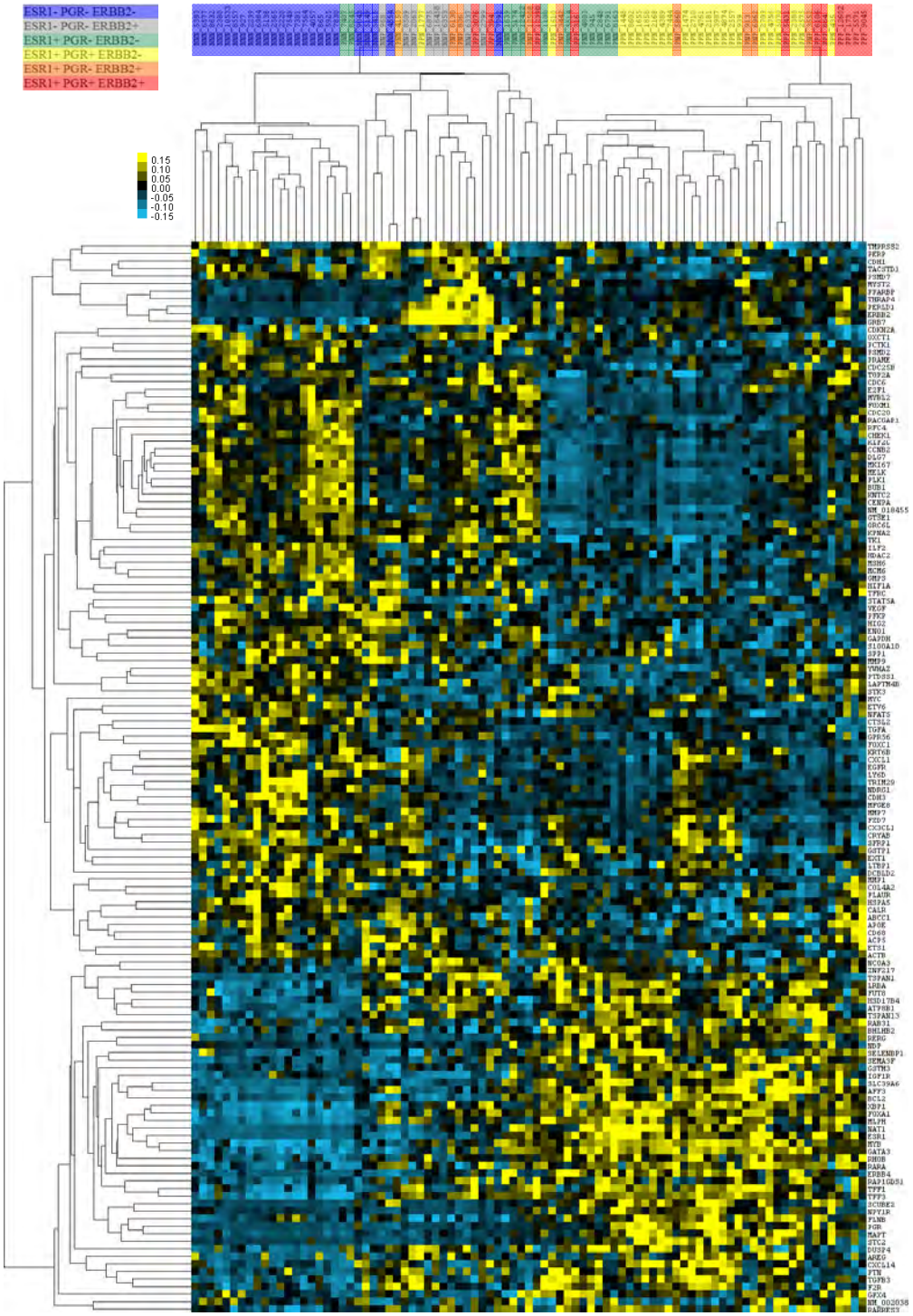

Figure 15. Unsupervised clustering of breast tumor samples. Tumor samples clustered into subgroups as previously seen in microarray experiments. Triple negative samples (TNTs) clustered together and away from the ESR+ERBB2± samples. Other major clusters included an ESR1+PGR±ERBB2- group and an ER+PR±ERBB2+ group.

Identification of regulated genes in TNT samples

To identify significantly altered genes, we performed Significance Analysis of Microarrays methods as previously described (Tusher et al., 2001) and found 73 genes that were differentially expressed between TNTs and other breast cancer subtypes with a False Discovery Rate of 1%. To further investigate the sets of genes detected by each panel, we performed a Venn diagram analysis. Differentially up-regulated genes and down-regulated genes in TNT samples, with at least 1.5 fold change, are shown in **Figure 16a** and **b**, respectively, for both the custom and standard cancer panels.

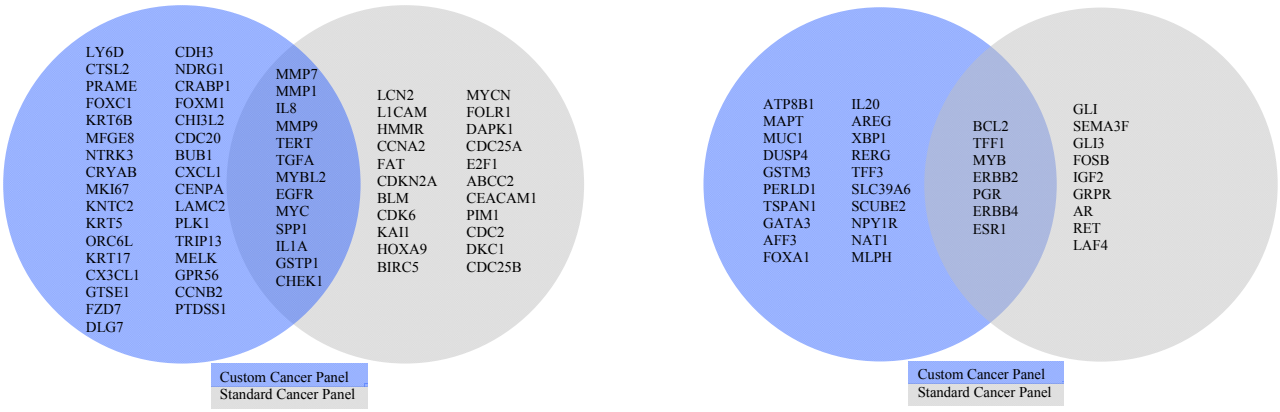

Figure 16. **a)** Differentially up-regulated genes in TNT samples with at least 1.5 fold change from both panels. **b)** Differentially down-regulated genes in triple negative (ESR1-PGR-ERBB2-) samples with at least 1.5 fold change from both panels.

Thus, we identified a subset of 73 genes (46 up-regulated 1.5-fold; 27 down-regulated 1.5-fold) from our custom DASL panel that were significantly different between the TNT subtype and other breast cancer subtypes. Using this subset of genes, hierarchical clustering was performed, as shown in **Figure 17**, which could readily separate the TNT samples from the others. Among the significantly decreased genes were ESR1 (ER), PGR (PR),

ERBB2 (HER2), and among the increased genes were EGFR, MMP7, FZD7, and MYC. The four upregulated genes EGFR (Schlange et al., 2007), MMP7 (Schwartz et al., 2003), FZD7 (Kirikoshi et al., 2001), and MYC (He et al., 1998), are all components or targets of the Wnt signaling pathway as identified by Ingenuity Pathway Analysis (IPA). Correlation of expression of EGFR, MMP7, and MYC with FZD7 expression across the 174 samples was highly significant (**Table 2**), suggesting a functional link with Wnt signaling. IPA identified several other significantly altered pathways as expected including estrogen signaling (p=1E-7), neuregulin signaling (p=1E-5), p53 signaling (p=1E-4), and cell cycle checkpoints (p=1E-3).

SYMBOL	*Correlation*	*p-value*
MMP7	0.57	3.91E-17
EGFR	0.57	1.14E-16
MYC	0.35	1.47E-06

Table 2. Correlation of expression of Wnt target genes with FZD7 expression across 174 FFPE DASL samples.

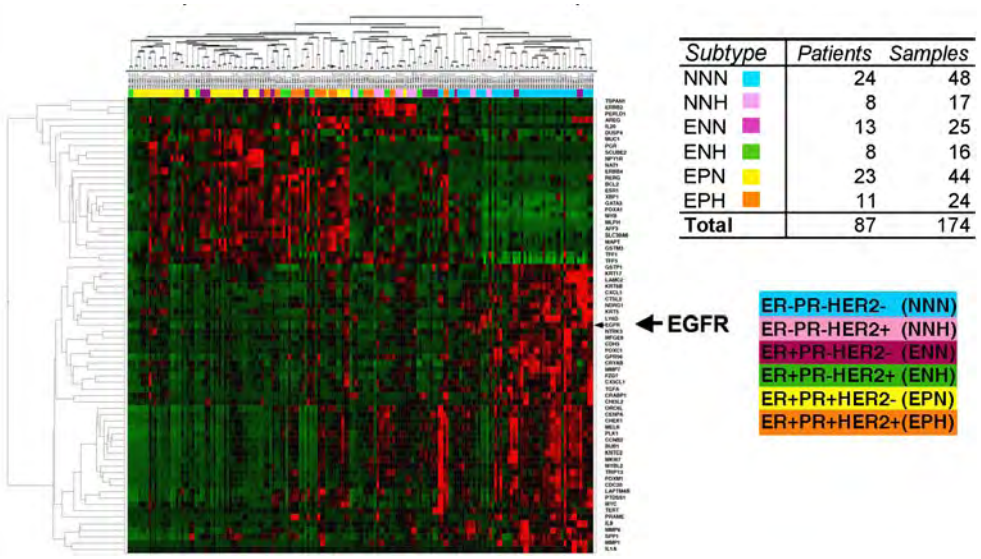

Figure 17: Hierarchical clustering of DASL analysis of 187 samples from 87 patients using genes that are significantly different between TNT and other subtypes.

It has been determined from a previous microarray study (Sorlie et al., 2003) that the following gene cluster is associated with the TNT subtype; CXCL1, CDH3, ANXA8, KRT5, TRIM29, KRT17, MFGE8, CX3CL1, FZD7, CHI3L2 and B3GNT5. Of the 11 genes, only 2 genes (ANXA8 and B3GNT5) are not in our custom panel. Eight of the 9 genes (except for TRIM29) were also upregulated in our TNT samples, further validating the DASL assay and the custom panel.

We also found that overexpression of EGFR (>1.5 fold increase over all other samples) occurred in only 4 of the 92 samples and all 4 were TNT samples, or 19% of the TNTs (**Figure 18**). TNTs have been shown to express EGFR by IHC.

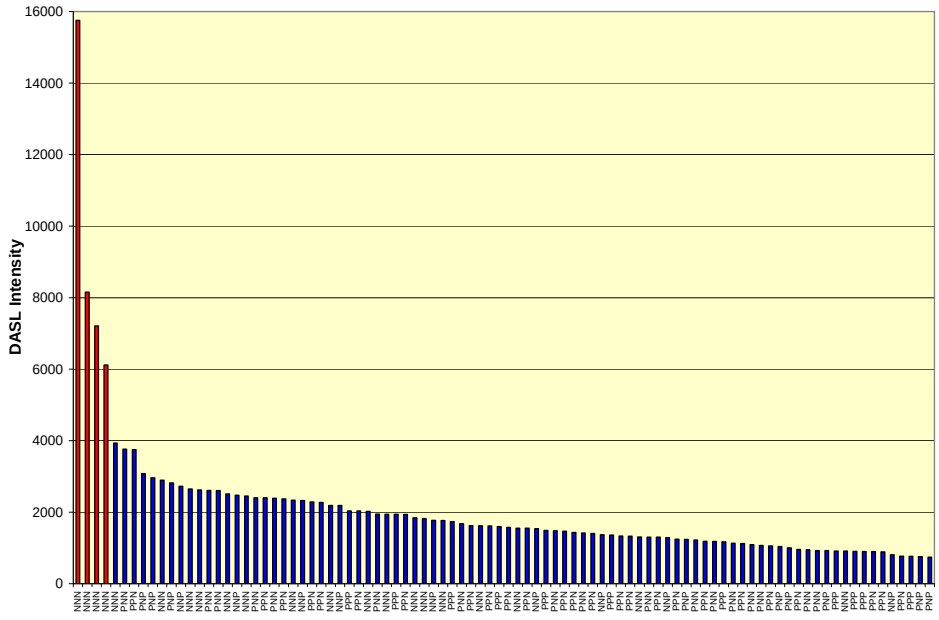

Figure 18. Expression of EGFR (highest to lowest) in all breast cancer tumor samples as measured in the DASL assay. IHC receptor status (NNN, PNN, PPN, PPP, PNP, NNP) is shown for each sample.

In one study (Nielsen et al., 2004), EGFR overexpression was found to occur in 38% of TNTs (8/21). More recently a poster was presented at the SABCS in which EGFR was detected by IHC in 57% (13/25) of TNTs but EGFR gene

amplification was only detected in 16% (4/25) of the TNTs (Pintens et al., 2007), which is more inline with our DASL data shown in **Figure 18** in which only 19% of the TNTs overexpressed EGFR.

Taken together, these data show, therefore, that we have validated both the DASL assay and our custom panel and that we have the infrastructure in place to robustly perform the DASL assay proposed in this project with high confidence and accuracy. Based on these data we recently received permission from ECOG to perform custom DASL analysis on FFPE tumor samples from the E2100 study. We are also seeking permission to perform DASL analysis on FFPE tumor samples from the E2197 Clinical trial, IBCSG BIG 1-98 Trial, as well as IBCSG Clinical Trial VIII & IX. This will put us in an excellent position to identify novel biomarkers and gene signature sets both prognostic and predictive.

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Pathology Core progress report for 07-08

Overview:

The activities of the pathology core include biobanking, processing and coordination of tissue acquisition and distribution. We receive frozen human serum and formalin fixed paraffin embedded tissue blocks from submitting institutions. Frozen serum is inventoried and placed in a -80°C freezer upon arrival. Paraffin blocks are stored by protocol and specimen number in block storage files. Data is entered into the HOG electronic data capture system on the day that the specimens are received. Data entered into the system includes: specimen number, date received, condition, and storage location. Paper records, without any protected health information, are stored (submission forms and pathology reports) in a locked file cabinet.

Processing of tissues and samples is determined by protocol. In all cases, we section formalin fixed paraffin embedded tissues to determine whether sufficient tumor exists in the block that was submitted. This quality control function allows us to request additional materials and give feedback to the submitting institution. For cases with small amounts of tumor in relation to the total amount of tissue present, the hematoxylin and eosin slide will be annotated so that tissue prepared for RNA analyses can be enriched for tumor.

We communicate with other investigators and Dr. Sledge through monthly conference calls, e mails and biannual in person meetings. We have had on site visits from administrative staff and work to be responsive to the needs of the reference laboratories.

CO1 Protocol
Through March 2008 samples from a total of 35 patients enrolled on clinical trial CO1 have been received. Among the 35 total patients, 12 were accrued to Arm A (Cytoxan/Adriamycin), 6 have been accrued to Arm B (Capecitabine), 3 accrued to Arm C (Vinorelbine) and 14 accrued to Arm D (Gemcitabine). During the 2007-2008 reporting period we distributed 9 pre-treatment and 5 off-treatment samples from Arm A and 13 pre-treatment samples from Arm D to the Proteomics Core for analysis. We have also cut and distributed 3 samples from Arm B to the Pharmacogenomics Core, who have prepared the RNA for themselves and the Genomics core.

CO2 Protocol
Through March 2008, a total of 5 patients have been accrued to this trial. Once additional specimens have been received, we will begin distributing serum and tissue specimens in accordance with the approved SOPs. Distribution is being held until 10 patient specimens have been accrued.

CO5 Protocol
Through March 2008, formalin fixed paraffin embedded archival specimens from 58 patients have had sufficient invasive tumor for analyses. During the current reporting period we distributed tissues only on patients treated with Capecitabine. Five 5-micron thick sections from 37 blocks (of 24 patients) were all sent to the Pharmacogenomics

Core for fluorescent in situ hybridization (FISH) analyses. Ten to twelve, 6 micron thick sections (from 47 blocks) were also sent to the same lab, for RNA extraction. RNA will be used by both the Pharmacogenomics Core and the Genomics Core.

Statistical Summary

- **Publications**

1. **Shen, C.**, Wang, Z, Shankar, G, Zhang, X, Li, L. A Hierarchical Statistical Model to Assess the Confidence of Peptides and Proteins Inferred from Tandem Mass Spectrometry. *Bioinformatics* 2008;24: 202-208.
2. **Shen, C.**, Breen, T.E., Dobrolecki, L.E., Schmidt, C.M., Sledge, G.W., Miller, K.D. and Hickey, R. J. Comparison of Computational Algorithms for the Classification of Liver Cancer using SELDI Mass Spectrometry: A Case Study. *Cancer Informatics* 2007; 3: 339-349.

- **Development**

1. R program developments for SELDI mass-spectrometry data analysis
2. Statistical methodological development for a general analysis strategy of microarray data with reliable control of false discovery rate (FDR) (first draft almost done).
3. Evaluation of the various statistical learning approaches for prediction model based on OMICS data.

- **Plans**

1. Data management and analysis group meeting to sort out details of data transfer and management issues
2. SELDI mass-spectrometry data analysis: identification of a panel of features for prediction of drug response/toxicity.
3. Gene expression data analysis: identification of a panel of genes for prediction of drug response/toxicity.
4. Develop prediction model to combine features in 2 and 3.